PCR Detection of *Babesia ovata* from Cattle Reared in Japan and Clinical Significance of Co-Infection with *Theileria orientalis*

**Running title:** Co-infection of *B. ovata* and *T. orientalis* in cattle

Thillaiampalam Sivakumar,¹ Michihito Tagawa,² Takeshi Yoshinari,¹ Adrian P. Ybañez², Ikuo Igarashi,¹ Yuzuru Ikehara,³ Hiroshi Hata,⁴ Seiji Kondo,⁴ Kotaro Matsumoto,² Hisashi Inokuma,² and Naoaki Yokoyama*¹

¹National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Inada-cho, Obihiro, Hokkaido, Japan
²Department of Clinical Veterinary Science, Obihiro University of Agriculture and Veterinary Medicine, Inada-cho, Obihiro, Hokkaido, Japan
³Research Center for Glycoscience, National Institute of Advanced Industrial Science and Technology, Tsukuba, Ibaraki, Japan
⁴Field Science Center for Northern Biosphere, Hokkaido University, Hidaka-gun, Hokkaido, Japan

*Corresponding author. Mailing address: National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Inada-cho, Obihiro, Hokkaido 080-8555, Japan. Phone: +81-155-49-5649; Fax: +81-155-49-5643; E-mail: yokoyama@obihiro.ac.jp
We describe here the clinical significance of co-infection with Theileria orientalis and Babesia ovata in cattle. Anemia status in a herd of dairy cattle in Japan was investigated in relation to infection with these parasites. Our findings indicate that while B. ovata infection might not be the primary cause of anemia in the cattle, it may contribute to the clinical development of anemia in animals co-infected with both B. ovata and T. orientalis.

Benign group of Theileria parasites (Theileria sergenti/buffeli/orientalis) and Babesia ovata are the only bovine piroplasms that are known to be endemic in Japan (1, 7, 13). T. orientalis is not considered to be highly pathogenic; however, clinical signs of anemia have sometimes been observed in cattle affected by this parasite (5, 8). In contrast, previous studies on splenectomized calves indicate that only immuno-compromised animals exhibit anemia-related clinical signs when they are infected with B. ovata (3). Both T. orientalis and B. ovata are transmitted to the bovine host by Haemaphysalis longicornis, which is an Ixodid tick vector (6). Therefore, mixed infections with the two parasites are expected to be very common in endemic areas (3).

Although several researchers have focused on the clinical consequences of infection with each of these parasites alone (3, 5), the pathobiology of mixed-infection with T. orientalis and B. ovata has not been evaluated. Infections with T. orientalis and B. ovata can be diagnosed by examination of Giemsa-stained thin blood smears under a light microscope. However, the lack of sensitivity and difficulty of species differentiation have limited the use of this technique (2). Therefore, PCR methods are employed because of their greater sensitivity and specificity (11). Although several PCR assays
have been developed for diagnosis of *T. orientalis* infection (8, 11), microscopy remains the only available technique for detecting *B. ovata*.

In the current study, we developed a novel PCR assay for detection of *B. ovata* in DNA samples extracted from infected bovine blood. A set of forward (5’-GATACGAGGCTGTCGGTAGC-3’) and reverse (5’-AGTATAGGTGAGCATCAGTG-3’) primers was designed to amplify a 504-bp fragment of the *B. ovata* *Apical membrane antigen 1* (*AMA-1*) gene (GenBank accession number: AB634843). One microliter of template DNA was added to 25 μl of a reaction mixture containing 2.5 μl of 10 × PCR buffer (Applied Biosystems, Branchburg, NJ, USA), 200 μM of each dNTP (Applied Biosystems), 0.8 μM of each primer, 1 U Taq polymerase (Applied Biosystems), and double distilled water. PCRs were conducted using a Veriti Thermal Cycler (Applied Biosystems). Optimized cycle conditions were used for the PCR. Briefly, 95°C for 5 min, followed by 45 cycles each of which consisted of a denaturing step at 95°C for 30 sec, an annealing step at 56°C for 1 min, and an extension step at 72°C for 1 min. After a final elongation step at 72°C for 10 min, agarose gels electrophoresis and ethidium bromide staining were followed by visualization of amplicons under UV light. The AMA-1 PCR amplified a 504 bp product from *B. ovata* DNA; no amplicons were produced using DNA derived from 11 other bovine blood pathogens (Fig. 1A). Furthermore, the assay was sensitive enough to detect 100 fg genomic DNA from an *B. ovata* (Miyake strain) *in vitro* culture (Fig 1B).

Blood samples were collected from 94 randomly selected dairy cattle from a dairy farm within the Shin-Hidaka district in Japan on the 19th of June, 2009. Microscopic examination of Giemsa-stained thin blood smears indicated that 29 (30.8%) and 3 (3.2%) of the animals were positive for infection with *T. orientalis* and *B.*
ovata, respectively. However, when DNA from the blood samples was screened using a previously described PCR method (MPSP-PCR) (8), and our newly developed AMA-1 PCR assay, 47 (50%) and 23 (24.5%) of the animals were found to be infected with T. orientalis and B. ovata, respectively (Table 1). All of the blood samples that were microscopy positive were also found to be positive using the two PCR assays (i.e. MPSP-PCR for T. orientalis; AMA-1 for B. ovata). Sequencing analysis confirmed that all of the B. ovata-specific amplicons had nucleotide sequences identical to the B. ovata AMA-1 gene (GenBank Accession number: AB634843). The degree of anemia in the animals was determined by red blood cell (RBC) counts, hemoglobin (Hb) concentrations, and hematocrits (HCT), using a Celltac (Nihon Kohden, Tokyo, Japan) automated hematological analyzer (8). An animal was considered to be anemic, if the RBC, Hb, or HCT value was less than $5 \times 10^6$ RBCs/$\mu$L, 8 g/dl, or 24%, respectively (4). Animals were categorized into four groups according to their infection profiles (Table 1). All of the non-infected animals had normal values for all three hematological parameters (i.e. RBC count, Hb and HCT); hence, other agents that could have contributed to the anemia among the animals could be ruled out. In addition, all the DNA samples were PCR screened for the presence of Anaplasma species as described previously (9, 12). Because only a single animal in the non-infected category was found to be infected with A. bovis, possible involvement of anaplasmosis as a cause of the anemia could be excluded. We found that the anemic animals were infected with T. orientalis alone or with T. orientalis and B. ovata (Table 1). Among the anemic cattle, all animals were positive for T. orientalis by microscopy, while B. ovata was detected on blood smear from a single animal. In agreement with a previous study (3), none of the animals that were only infected with B. ovata were anemic. The anemia rates among
the co-infected animals (42.9%) were significantly higher than in animals infected with
*T. orientalis* (18.2%) alone (Table 1). Among the anemic animals, three exhibited low
values for all three hematological parameters; two of these animals were found to be
co-infected with both of the parasites (data not shown). The mean RBC count of the
animals (n=33) infected only with *T. orientalis* (5.90 × 10⁶/μl) was lower than that of
the non-infected animals (7.29 × 10⁶/μl), while the RBC count of the co-infected
animals (n=14) (5.64 × 10⁶/μl) was similar to that obtained for those animals infected
with *T. orientalis* alone (Table 2). In contrast, a significant reduction of the mean Hb
count was only observed in the co-infected animals (10.3 g/dl), when compared
with the non-infected animals (11.6 g/dl) whereas *T. orientalis* infection alone did not
alter the mean Hb concentration (11.2 g/dl). Similar observations was also made for the
mean HCT value, in which only the co-infected animals (27.8%) showed a lower HCT
value than the non-infected animals (32.0%) (Table 2).

Based on these findings, we suggest that *B. ovata* might be a risk factor for the
induction of clinical anemia when animals are co-infected with *T. orientalis*, although *B.
*ovata* is unlikely to be the sole agent causing anemia in the cattle population in endemic
regions. In common with other *Babesia* parasites, *B. ovata* induces intra-vascular
hemolysis as was evident from the hemoglobinuria detected when splenectomized cattle
were infected with the parasite (3). In contrast, the anemia induced by *T. orientalis* is
thought to be due to erythrophagocytosis of red blood cells (10). Therefore, we assume
that the combination of these two different mechanisms for anemia could have
potentiated the serious development of clinical anemia among the animals that were
co-infected with *B. ovata* and *T. orientalis*. In this report, we have described the effects
*T. orientalis* and *B. ovata* infections have on the anemia status of a cattle population.
Further studies, using a larger sample size are now necessary to confirm our findings. In addition, *in vivo* experiments to study the effects of co-infection with both parasite species in susceptible animals are a high priority.
ACKNOWLEDGMENTS

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REFERENCES


Figure 1. Babesia ovata-specific AMA-1 PCR assay development. A new PCR method was developed for the diagnostic detection of B. ovata, based on the B. ovata AMA-1 gene. Panel A: PCR specificity. Lanes 1-13 represent genomic DNA samples from B. ovata, B. bigemina, B. bovis, Theileria annulata, T. orientalis, Trypanosoma brucei gambiense, T. evansi, T. theileri, Aanaplasma marginale, A. bovis, A. centrale, A. phagocytophilum, and normal bovine blood, respectively. Panel B: PCR sensitivity. 10-fold serial dilutions of B. ovata genomic DNA extracted from in vitro culture. Lanes 1-8 represent 10 ng/μl, 1 ng/μl, 100 pg/μl, 10 pg/μl, 1 pg/μl, 100 fg/μl, 10 fg/μl, and 1 fg/μl, respectively. An uninfected bovine DNA sample was included as a negative control (lane 9). M, 100-bp DNA marker ladder appears in both panels.
Figure 1. Sivakumar et al.
Table 1. Summary of diagnostic results obtained for 94 field grazing cattle using *T. orientalis* MPSP-PCR and *B. ovata* AMA-1-PCR assays

<table>
<thead>
<tr>
<th>Infection type*</th>
<th>Positive animals (%)</th>
<th>Anemic animals</th>
<th>Anemia rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-infected</td>
<td>38 (40.4)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>T. orientalis</em> **</td>
<td>33 (35.1)</td>
<td>6</td>
<td>18.2</td>
</tr>
<tr>
<td><em>B. ovata</em>*</td>
<td>09 (9.6)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>T. orientalis &amp; B. ovata</em></td>
<td>14 (14.9)</td>
<td>6</td>
<td>42.9</td>
</tr>
</tbody>
</table>

*Animals were categorized into 4 groups which consisted of non-infected, *T. orientalis* infected, *B. ovata* infected, or co-infected animals, based on the infection types.

** Animals infected with a single parasite species
Table 2. Mean red blood cell count, hemoglobin and hematocrit values for the different categories of animals

<table>
<thead>
<tr>
<th>Infection type</th>
<th>Mean value ± Confidence interval</th>
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<tbody>
<tr>
<td></td>
<td>RBC count (x 10^6/μl)</td>
</tr>
<tr>
<td>Non-infected</td>
<td>7.29 ± 0.29</td>
</tr>
<tr>
<td>T. orientalis</td>
<td>5.90 ± 0.35 *</td>
</tr>
<tr>
<td>B. ovata</td>
<td>7.24 ± 0.63</td>
</tr>
<tr>
<td>T. orientalis &amp; B. ovata</td>
<td>5.64 ± 0.56 *</td>
</tr>
</tbody>
</table>

* Denotes statistically significant reductions in the mean values obtained compared to the non-infected animals (P < 0.01).